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ONTARIO AGRICULTURAL COLLEGE.

BULLETIN 169.

Legume Bacteria

Further Studies of the Nitrogen Accumulation in the Luguminosæ

BY

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LEGUME BACTERIA.

FURTHER STUDIES OF THE NITROGEN ACCUMULATION IN THE LEGUMINOSÆ.

By S. F. Edwards and B. Barlow.

Studies of the nitrogen accumulating bacteria, *Ps. radicicola*, have been in progress in this laboratory during a period of about five years. The results of investigation up to 1906 were compiled and published by Harrison and Barlow in the Proceedings of the Royal Society of Canada, 1906, and in the Centralblatt für Bakteriologie, II Abte., Vol. 19, 1907.

1. *Ps. radicicola* from nodule of *Medicago sativa*, Alfalfa, showing both minute rods and large branched cells. Amyl-Gram stain. X 1,000.

2. *Ps. radicicola* from nodule of *Medicago sativa*, Alfalfa. Bacteroidal cells only. Carbol-fuchsin stain. X 1,500.

Briefly the results embodied are as follows:

There were examined upwards of thirty foreign economic species of Leguminosæ of the sub-order *Papilionaceæ*, exclusive of some twenty-four species and varieties of the genus *Vicia*, also a number of native species. Nodules were found on the roots of all of these with the exception of two species, *Cicer arientum*, and *Galega officinalis*. Plants

of the sub-order *Cæsalpinæ* were examined and no nodules were found on the roots of *Gimnocladus*, *Gleditschia* or *Cercis canadensis*, but mycorhiza were present in all cases.

Ps. radicicola was isolated from the following host plants:

TRIFOLIÆ:
Medicago sativa
Melilotus alba
Trifolium incarnatum
Trifolium pratense
Trifolium repens.

HEDYSAREÆ:
Desmodium acuminatum
Desmodium canescens
Desmodium nudiflorum.

VICIEÆ:
Vicia villosa
Lathyrus sativus
Pisum sativum.

PHASEOLEAE:
Glycine hispida
Apios tuberosa
Phaseolus vulgaris.

3. *Ps. radicicola* from nodule of *Medicago sativa*, Alfalfa. Only small rod forms were present. Saturated alcoholic gentian violet stain. X 1,000.

4. *Ps. radicicola* from *Medicago sativa*, Alfalfa. Culture on ash-sugar-agar, showing polar flagella. Saturated alcoholic gentian violet stain. X 1,000.

MEDIA FOR GROWTH OF *Ps. radicicola.*

Various media were tried, and it was found that the organism grew uxuriantly in an ash-sugar-agar, prepared as follows:—

"To 1000 c.cm. of cold water, either tap or distilled, add 2.5 g. to 25 g. wood ash; stir in and filter at once, or at any time up to one-half hour. Or the ash may be added to boiling water, boiled a minute or two and filtered at once. The filtering may be done through a tuft of absorbent cotton at the pump. The filtrate should be clear and colourless and should be more alkaline to phenolphthalein as the amount of ash or the time before filtration is increased. The acidity at this point was found to vary from very faintly alkaline to-17.5° acid to phenolphthalein. At this point agar is added in amount from 0.5% to 1.5%. The agar is added to the ash filtrate and the mixture boiled over the

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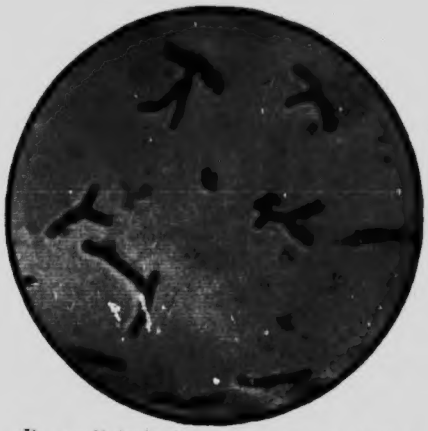
flame with stirring, or heated in flowing steam or the autoclave until the agar is all dissolved. Maltose is now added in amount from 0.4% to 4%. The medium is heated for a few minutes or allowed to stand until the sugar is thoroughly dissolved, when the medium is filtered through absorbent cotton at the pump. Sterilization may be affected either in flowing steam or in the autoclave.

"If a liquid medium is desired, the same procedure is followed, except that the agar is omitted."

Ashes from maple or mixed beech and maple, from elm and from tamarack were used with equally favourable results.

5. *Ps. radicicola* from nodule of *Melilotus alba*, White Sweet Clover. Cuneate and branched cells. Amyl-Gram stain. X 1,500.



6. *Ps. radicicola* from nodule of *Melilotus officinalis*, Yellow Sweet Clover. Small rods and much branched cells. Amyl-Gram stain. X 1,500.

This medium contains from the ash, all the mineral elements required for bacterial growth, the sugar is the source of energy, and the nitrogen is secured from the atmosphere. The nitrogen accumulating power of the organism is thus maintained.

The tabulated summary of media used follows:—

Tabulated Summary of Media.

Water 100.

	Agar None.							Agar 1 per cent.								
Medium R..	31	32	33	45	42	49	50	35	44	46	48	34	46.1	36	37	47
Ash.....	1	1	1	1	1.5	1	2	1	1	1	2	1	1	1	1	2
Maltose....	....	..	.....	2	.....	1	1	2	2	1	2	....	.. .	2	0.5	1
Sucrose.....	....	2	2	.....	.....	.....	.....	...	..	..	..	2	1	...	2	.....
KH_2PO_4...	....	....	0.5	0.2	0.5	2	2	...	..	..	..	....	....	0.5	0.5	1
To Litmus..	-11°	-10°	+11°	+4°	Alk.	+28°	+28°	-5°		N.	N.	-5°	-5°	+	+	+2°
To P'thalein	-8°	-6°	+31°	+19°	.....	+93°	+63°	-2°	..	N.	N.	-2°	-5°	+	+	+18°

N—Neutral. Alk.—Alkaline.

Tap Water 100.

Medium R.....	74	76	77	78	80	81	82	83	51
Agar.....	1.5	1.2	1.5	1	2	1.5	1.2	1.2	0.75
Maltose.....	2	2	2	1	1	1	.....	.25	1
Ash.....	.66	.66	.75	1	.5	1	.25	.25	.5
To Litmus.....	Alk.	.....	.....	−10°	.....	.....	.....	.....	.....
To P'phthalein.....	Neut.	−2°	Alk.	−2°	.....	.....	.....	.....	.....

Distilled Water 100; Maltose 1 per cent.

Agar None.			Agar 1 per cent.			Agar 1.5 per cent.		
Ash	Reaction	Stock R	Ash	Reaction	Stock R	Ash	Reaction	Stock R
0	+1°	52	0	+3°	53	1	−5°	69
0.5	+1°	54	0.5	−2°	55	1.5	−7°	70
1.0	−2.5°	56	1.0	−2°	57	0.5	−4°	71 Tap W.
1.5	−3°	58	1.5	−2°	59	1.0	Alk.	72 Tap W.
2.0	−4.5°	60	2.0	−2°	61			
2.5	−6°	62	2.5	−2°	63			
0.5	Faintly Alk.	68	3	−8°	64			
0.5	.....	73	4	−13°	65			
0.5	.....	79	5	−21°	66			

Ash 1, Maltose 1, Agar 1.			Ash—Maltose—Water.				
Water	Reaction	Stock	Water	Ash	Maltose	Reaction	Stock
100 tap	+1°	R 87	100 dist.	0.5	0.5	−0.5°	R 90
100 dist.	.....	R 97	100 dist.	0.5	0.5	−0.2°	R 91
100 dist.	−2°	R 98	100 tap	1	1	.....	R 92
100 dist.	.....	R 99	100 tap	1	1	.....	R 93
100 dist.	.....	R 100	100 dist.	1	1	Neut.	R 95
100 tap	Neut.	R [illegible]	100 tap	1	1	.....	R 96
100 tap	.....	[illegible]					

Similar media were used for growing legumes in 1½ L. Erlenmeyer flasks. Seeds of legumes were secured from the pods under aseptic conditions, and grown in Erlenmeyer flasks for studies in inoculation with pure cultures. Figs. 31 and 3[illegible].

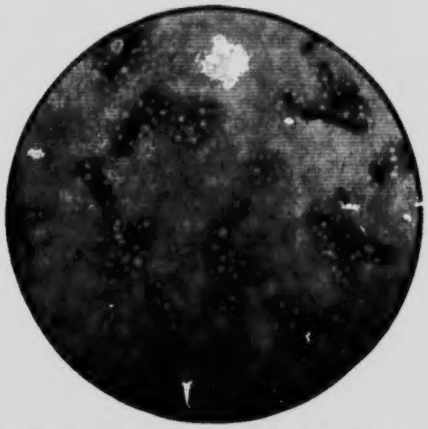
Since the publication of the previous work, we have examined the nodules and found bacteria present in additional host plants, as follows:

TRIFOLIÆ:
Medicago lupulina
Melilotus officinalis
Trifolium hybridum
Trifolium procumbens.

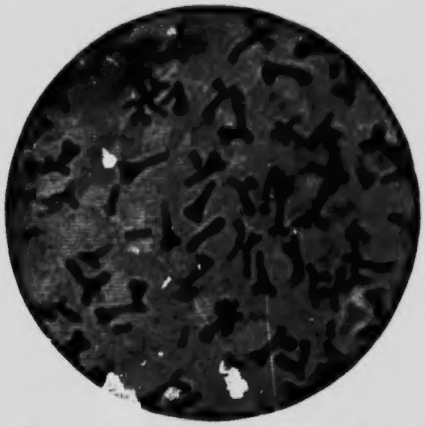
GALEGEÆ:
Caragana frutescens
Robinia pseudacacia
Robinia viscosa.

VICIEÆ:
Vicia faba
Vicia americana
Lathyrus sylvestris
Lathyrus odoratus.

PHASEOLEÆ:
Phaseolus multiflorus.



7. *Ps. radicicola* from nodule of *Melilotus officinalis*, Yellow Sweet Clover. Rods and branched forms, the latter with very irregularly distributed protoplasm. Carbol-fuchsin stain. X 1,000.



8. *Ps. radicicola* from nodule of *Vicia villosa*, Hairy Vetch. Eosin-methylene blue stain. X 1,500.

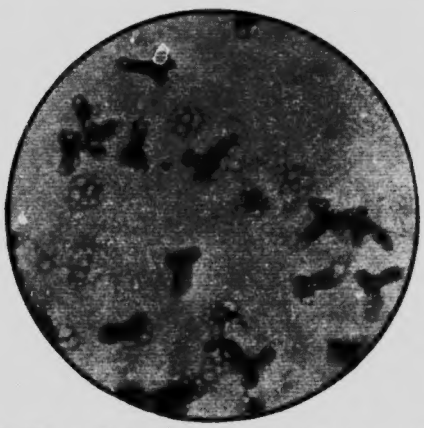
The organism was isolated, and pure cultures prepared from all but *Trifolium procumbens*. Cultures were also isolated from ten of the hosts from which cultures were previously isolated.

Investigation in media preparation was continued, and it was found that for general studies best results were obtained on media prepared in the proportion of water 100, ash .4% to 1%, maltose .4% to 1%, agar .4% to 1.5%.

Media were prepared using other substances than maltose as the source of energy for the organism. The table of modified ash-agar media, and results of growth, follow.—

Media R 129—R 146.

Stock R.	Water.	Ash.	Agar.	Sugar.	Reaction.
129.....	100	.5	1.5	.....	-7° alkaline.
130.....	"	"	"	Maltose, 1.	
131.....	"	"	"	Mannit, .5.	
132.....	"	"	"	Dextrose, 1.	
133.....	"	"	"	Levulose, 1.	
134.....	"	"	"	Dextrin, c. p. 1.	
135.....	"	"	"	Inulin, .25.	
136.....	"	"	"	Gum tragacanth, .5	
137.....	"	"	"	Amygdalin, .25.	
138.....	"	"	"	Asparagin, .25.	
145.....	"	"	"	Dextrin, com'l 1.	
146.....	"	"	"	" " Glycerine, 1.	



9. *Ps. radicicola* from nodule of *Vicia Americana*, "Wild Pea," from Big Creek, B,C. Carbol-fuchsin stain. X 1,000.

10. *Ps. radicicola* from nodule of *Vicia faba*, Horse Bean. Amyl-Gram stain. X 1,500.

Plate cultures, 17 days at 25° C., in ash-water-agar modified with sugar.

Colonies none.

R. 133, levulose, 1%.

Colonies small, 1 m.m., not viscid.

R 129, Ash-water-agar alone.

Colonies small, 2 m.m.

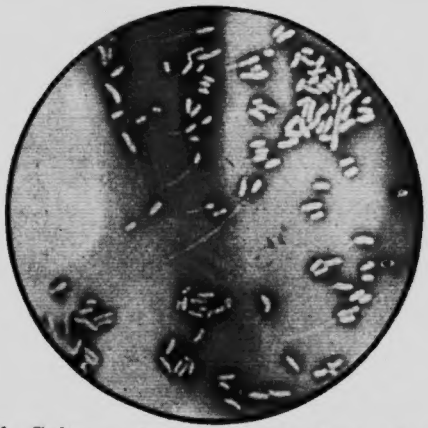
R 136, Gum tragacanth, 1/2%, all viscid.
R 137, Asparagin, 1/4%, only 1,400 viscid.
R 135, Inulin, 1/4%, only 1,300 viscid.

Colonies large, 3-10 m.m., deep 2-4 m.m., all very viscid.

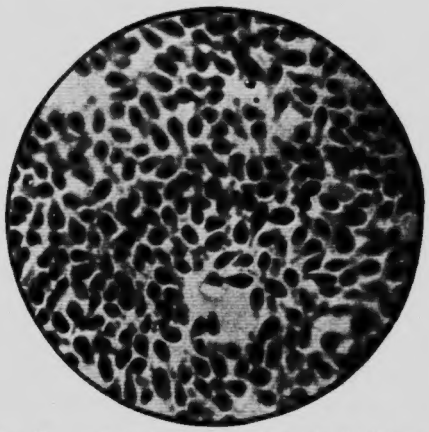
R 131, 1/2% mannit	2-4	m.m.	in 4	days	surface colonies.
	4-7	"	" 7	"	" "
	5-10	"	" 17	"	" "
	2-4	"	" 17	"	" "
R 132, dextrose, 1%	6-12	"	" 17	"	" "
R 137, amygdalin, 1/4%...	4-10	"	" 17	"	" "
R 130, maltose, 1%	3	"	" 17	"	" "
R 134, Dextrin, 1%	3	"	" 17	"	" "

The cultures used in the above plate cultures were:—*Vicia villosa*, (stock culture), *Vicia villosa*, direct from nodule of host plant, *Trifolium pratense*, direct from nodule of host plant, *Trifolium pratense*, (stock culture), *Pisum sativum*, (stock culture).

Five plates were made in each of the ten media. Wherever growth occurred, it was typical of *Ps. radicicola*, as was the morphology of the organisms in stained preparations



11. Culture of *Ps. radicicola* from *Vicia villosa*, Hairy Vetch, on ash-maltose-agar showing flagella. Saturated alcoholic gentian violet stain. X 1,000.



12. *Ps. radicicola* from nodule of *Trifolium pratense*, Red Clover. Amyl-Gram stain. X 1,000. Compare with No. 30.

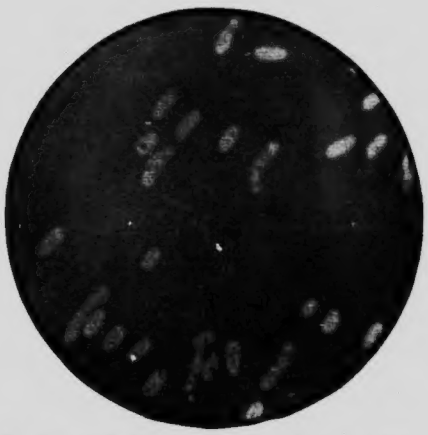
Streak Culture on Agar, 25° C. Character of the growth was typical of *Ps. radicicola*, the rapidity and abundance being stated as follows: Growth abundant in 10 days—Maltose, Mannit, Dextrose, Amygdalin; growth less abundant—Dextrin; growth scant—Inulin, asparagin, Gum Tragacanth, no sugar; growth none—Levulose.

Time of appearance. No colonies were visible to the eye in 48 hours. Colonies were visible to the eye in 3 days in all plates of the series except in maltose, dextrin and dextrose. In maltose and dextrin colonies were visible in 3 to 4 days; in dextrose in 7 to 9 days.

From the results it is seen that maximum growth in 17 days occurred in dextrose, mannit, and amygdalin, was very scant in asparagin and inulin, and was nil in levulose.

The effect of the different sugars in liquid media was also tried. The media consisted of ash-water, stock E 87, modified as follows:—

Stock E.	Water.	Ash.	Sugar.
87	100	.5	None.
87 a	"	"	Glycerine, .5.
87 b	"	"	Mannit, .5.
87 c	"	"	Dextrose, 1.
87 d	"	"	Maltose, 1.
87 e	"	"	Levulose, 1.
87 f	"	"	Raffinose, .5.



13. *Ps. radicicola* from *Trifolium pratense*, Red Clover. From colony on ash-maltose-agar, R 112. Saturated alcoholic gentian violet stain. X 2,000.

14. *Ps. radicicola* from the nodule of *Phaseolus multiflorus*, Scarlet Runner Bean. Rod forms only in nodule. Saturated alcoholic gentian violet stain. X 1,500.

The cultures used were stock cultures on ash-maltose-agar from the following host plants:—*Trifolium pratense*, red clover; *medicago sativa, alfalfa; Trifolium hybridum*, alsike clover; *Pisum sativum*, peas; *Vicia villosa*, vetch; *Lathyrus odoratus*, sweet pea; *Phaseolus vulgaris*, bean; *Robinia pseudacacia*, locust,

The results follow:—

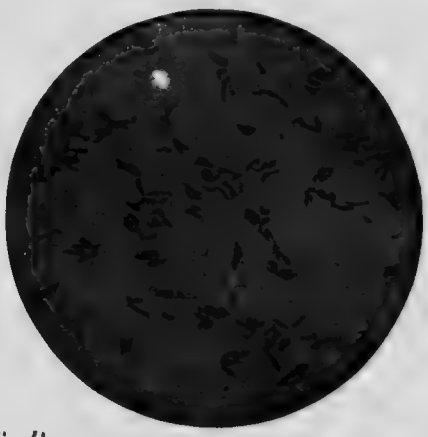
Four days at 25° C.—

E 87 (no sugar). No growth of any cultures.

E 87 a, (glycerine). No growth—Alfalfa, red clover. Slight cloudiness—All others.

E 87 b, (mannit). No growth—Alfalfa. Slight cloudiness—All others.
E 87 c, (dextrose). No growth—Alfalfa, peas, locust. Slight cloudiness—All others.
E 87 d, (maltose). No Growth—Vetch, locust. Slightly cloudy—Alfalfa. Very markedly cloudy—All others.
E 87 e, (levulose). No growth—All cultures.
E 87 f, (raffinose). Slightly cloudy—All cultures.
Twenty-two days at 25° C.—
E 87 (no sugar). Very slight cloudiness of all cultures.
E 87 a, (glycerine). Slightly cloudy—Alfalfa, beans, alsike clover. Markedly cloudy—All other cultures.
E 87 b, (mannit). Slightly cloudy—Alfalfa, locust. Markedly cloudy, with copious sediment—All other cultures.

15. *Ps. radicicola* from *Phaseolus vulgaris*, Garden Bean. From colony on ash-maltose-agar. Saturated alcoholic gentian violet stain. X 1,000.



16. *Ps. radicicola* from nodule of *Robinia pseudacacia*, Black Locust. Carbol-fuchsin stain, X 1,000.

E 87 c, (dextrose). Slightly cloudy—Alfalfa, locust, vetch, peas. Densely cloudy—All other cultures.
E 87 d, (maltose). No growth—Locust. Al other cultures very densely cloudy with copious sediment, and pellicle in some cases.
E 87 e, (levulose). No growth in any cultures.
E 87 f, (raffinose). Slightly cloudy—Alfalfa, locust, beans, peas, Markedly cloudy—All others.

The results show that of the sugars tried maltose gave the most abundant growth in liquid media. The unsuitability of levulose in media for *Ps. radicicola* was shown as in the solid media tests, there being no growth in any cultures.

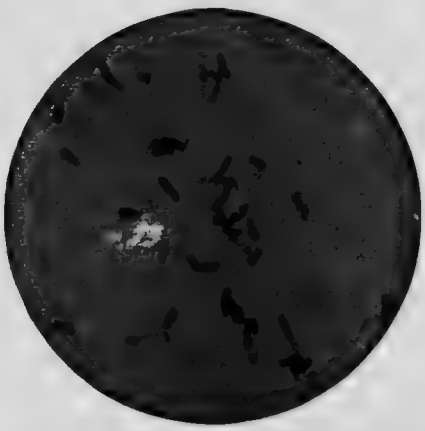
Isolation of *Ps. radicicola* from the Nodule.

The technique of isolation from the nodule was varied slightly from the method used in the previous studies, the exact procedure being as follows:—

The plant is dug, care being observed not to break off the nodules, and the roots placed immediately in clean, cool water. Parts of the plant are preserved for identification, and nodules removed to killing and fixing solutions preparatory to sectioning.

A nodule is removed from the roots, washed thoroughly under the tap and immersed in 50 c.cm. to 100 c.cm. of the following solution:—

Hydrochloric acid, sp. gr. 1.20	2.5	c.cm.
Mercuric chloride crystals	1	g.
Water, distilled or tap	1,000	c.cm.



17. *Ps. radicicola* from nodule of *Glycine hispida*, Soy Bean. Mostly small rod forms. Carbol-fuchsin stain. X 1,500.

18. *Ps. radicicola* from *Lathyrus odoratus*, Sweet Pea. 25 day old culture on ash-maltose-agar. Saturated alcoholic gentian violet stain. X 1,500.

The nodules may remain immersed for 2-3 minutes unless in the case of large nodules, as from soy beans, when a longer immersion does no harm. The nodule is taken with flamed forceps and placed between folds of filter paper moistened with the above solution. The nodule is held with flamed and cooled forceps and broken open with a flamed platinum knife or small scalpel. A flamed needle is thrust into the middle of the broken surface, gently rotated, and then touched into a drop of sterile water in a sterile Petri dish. Three loopfuls of this inoculated water are transferred to a second drop of sterile water in the same dish. A third drop in the same dish is inoculated from the second in like manner. In a second Petri dish, three drops of sterile water are inoculated in the same succession, the first transfer being from drop 3 in the first dish. Liquified ash-maltose-agar, cooled to near its solidifying temperature,

is mixed with the several drops of inoculated water, and the plates when solidified may be placed at a temperature of 20° or 25° C.

At the same time at least three smears should be made from the nodule for staining.

Hanging drop examinations are also made, crushing a particle of the bacteroidal tissue in a drop of distilled water on a cover glass.

Studies of the cultural and morphological characteristics were continued, and the observations previously noted were confirmed. Figs. 1 to 30.

In a few plants, notably *Pisum sativum*, when the nodule was broken open the bacteroidal tissue was observed to be mucilaginous. Hanging drop preparations showed some large branched cells, non-motile, and some small or minute rods swiftly and actively motile, darting, whirling, and tumbling. Some of the mucilaginous tissue, spread on a slide and stained with saturated alcoholic gentian violet, gave a typical flagella stain, showing the cells and whips unstained in the densly stained background.

19. *Ps. radicicola* from *Medicago sativa*, Alfalfa. Colony on ash-maltose-agar. X 200.

20. *Ps. radicicola* from *Lathyrus odoratus*, Sweet Pea. Colony on ash-maltose-agar. X 100.

Viability of *Ps. radicicola*.

Observations on the viability of *Ps. radicicola* on ash-maltose me. have been continued. It would appear that on favourable media the ganism is long lived. The results obtained in this laboratory are embodied in the following table. The cultures were grown a short time at 20° or 25° C., and were then kept at the temperature of the laboratory. These same cultures were all successfully transferred to various other media more than once in the interval recorded in the table, and gave a prompt and characteristic growth on favourable media. The transfers in the table were to ash-maltose-agar in all cases, and to

ash-maltose-water in some cases. The growth, morphology, and staining reactions were carefully observed, and were characteristic of *Ps. radicicola*. The same is true of colonies which developed in plate cultures in ash-maltose-agar made from certain of these cultures, after a lapse of nearly three and one-half years in one case. The growth was abundant and mucilaginous, the cells were actively motile in hanging drops, and stained with saturated alcoholic gentian violet they showed single polar flagella. Occasional branched forms were seen from agar cultures and were frequent in liquid media.

Viability of Ps. Radicicola in Cultures at Room Temperature.

Data March, 1908.

Isolated from.	Stock number.	Cultivated in water 100% with				Alive after	
		Ash.	Maltose.	KH_2PO_4.	Agar.	Years.	Days.
		%	%	%	%		
Scarlet Runner Bean (*Phaseolus multiflorus*)	R 107	0.5	1	0	1	1	137
	R 107	0.5	1	0	1	1	235
Bean (*Phaseolus vulgaris*)	R 35	1	2	0	1	3	146
	R 35	1	2	0	1	3	140
	R 35	1	2	0	1	1	253
	R 35	1	2	0	1	1	259
Red Clover (*Trifolium pratense*)	R 112	0.2	0.5	0	1.5	1	27
	R 112	0.2	0.5	0	1.5	1	125
	R 72*	1	1	0	0.9	1	301
	R 72	1	1	0	0.9	1	91
Sweet Pea (*Lathyrus odoratus*)	R 105	0.5	1	0	1	1	148
	R 105	0.5	1	0	1	1	246
Alfalfa (*Medicago sativa*)	R 54	0.5	1	0	0	3	43
	R 54	0.5	1	0	0	1	146
	R 54	0.5	1	0	0	1	156
	R 34	1	0**	0	1	1	340
	R 54	0.5	1	0	0	1	123
Soja Bean (*Glycine hispida*)	R 106	0.5	0.6	0.03	0	1	221
Horse Bean (*Vicia faba*)	R 107	0.5	1	0	1	1	117
	R 107	0.5	1	0	1	1	209
	R 104	1	1	0	1	1	210
Hairy Vetch (*Vicia villosa*)	R 54	0.5	1	0	0	3	43
	R 44*	1	2	0	1	1	353
White Clover (*Trifolium repens*)	R 36	1	1	0.5	1	1	181
Peas (*Pisum sativum*)	R 104	1	1	0	1	1	244
	R 87	1	1	0	1	1	343
	R 87	1	1	0	1	1	341
	R 36	1	1	0.5	1	1	175
	R 36	1	1	0.5	1	2	26

Data March, 1908.—*Continued.*

Isolated from	Stock number.	Cultivated in water 100% with				Alive after	
		Ash.	Maltose.	$K H_2 PO_4$.	Agar.	Years.	Days.
Bitter Vetch (*Lathyrus sativus*)	R 118	0.2	0.5	0	1.5	0	356
	R 112	0.2	0.5	0	1.5	1	125
	R 112	0.2	0.5	0	1.5	1	27
	R 35	1	2	0	1	2	159
	R 35	1	2	0	1	1	335
Black Locust (*Robinia pseudacacia*) ...	R 105	0.5	1	0	1	1	198
	R 105	0.5	1	0	1	1	296
Flat Pea (*Lathyrus sylvestris*).....	R 104	1	1	0	1	1	243
	R 104	1	1	0	1	1	335
Wild Bean (*Apios tuberosa*).....	R 36	1	1	0.5	1	2	47
Tick trefoil (*Desmodium nudiflorum*)...	R 79	1	1	0	0	1	274
Siberian Pea Tree (*Caragana frutescens*)	E 31	1	0.5	0	1	2	126
Red Clover (*Trifolium pratense*).....	R 104	1	1	0	1	2	139
	E 30***	1	0.5	0.5	1	1	347
Soy Bean (*Glycine hispida*).....	E 30	1	0.5	0.5	1	1	358
Sweet Pea (*Lathyrus odoratus*).....	E 31	1	0.5	0	1	2	37
Garden Pea (*Pisum sativum*).....	E 30	1	0.5	0.5	1	2	104
Alsike Clover (*Trifolium hybridum*)....	R 104	1	1	0	1	2	139
Bitter Vetch (*Lathyrus sativus*)	E 30	1	0.5	0.5	1	2	125
Flat Pea (*Lathyrus sylvestris*).....	E 30	1	0.5	0.5	1	2	125
Alfalfa (*Medicago sativa*).....	E 30	1	0.5	0.5	1	2	35
Black Medick (*Medicago lupulina*)	E 30	1	0.5	0.5	1	2	125
Horse Bean (*Vicia faba*)	E 30	1	0.5	0.5	1	2	15
Black Locust (*Robinia pseudacacia*) ...	E 30	1	0.5	0.5	1	1	358
Honey Locust (*Robinia viscosa*)	E 30	1	0.5	0.5	1	2	121
Dutch White Clover (*Trifolium repens*)	E 31	1	0.5	0	1	2	98
"Wild Pea" (*Vicia Americana*).....	E 86†	0.4	0.5	0	1	0	121
Garden Bean (*Phaseolus vulgaris*)	E 31	1	0.5	0	1	2	117
Scarlet Runner Bean (*Phaseolus multiflorus*).....	E 32	0.2	0.5	0	1.5	1	220
Hairy Vetch (*Vicia villosa*)	R 104	1	1	0	1	2	133
Sweet White Clover (*Melilotus alba*)...	E 30	1	0.5	0.5	1	2	123

* Cultures made from this culture were distributed for inoculation of seed in 1906
** Sucrose 2%.
*** Isolated from dried plants from Medicine Hat, Alta.
† Plants from Big Creek, B.C.

The studies on viability in cultures of ash-sugar-agar will be continued. For the purpose it is desirable to have receptacles for the cultures, holding an ample quantity of medium, and so constructed as to reduce the rate of evaporation to a minimum. Two forms of such flasks, designed in this laboratory, made of Jena glass, are shown in Figs. 39

and 40. Determinations showed that in the smaller flask, half filled, 40 to 50 years would elapse before evaporation of the agar was complete.

Effect of Desiccation.

Limited studies were made on the effect upon *Ps. radicicola* of desiccation on seed, on glass, and on filter paper.

Desiccation on Seeds. A culture was taken from the stock as prepared and ready to send to farmers for seed inoculation, was shaken with sterile water, 600 c.cm., until the agar was thoroughly broken up.

Dilutions were made in sterile water, and ash-maltose-agar plates poured to determine the number of organisms in the culture.

Portions of seed purchased in the local seed stores were handled as follows:—

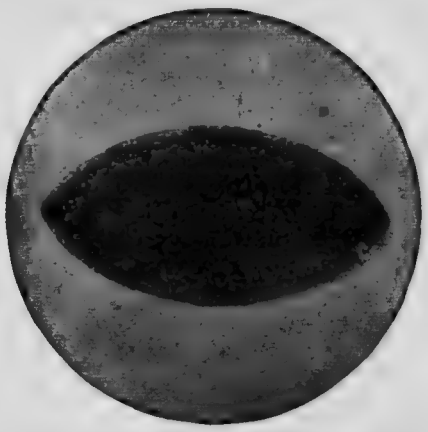
Portion A.—One pound seed inoculated with 10 c.cm. of stock culture as diluted above.

Portion B.—One pound seed uninoculated.

Portion C.—One pound *sterilized* seed inoculated with 10 c.cm. of stock culture diluted as above.

Portion D.—One pound *sterilized* seed uninoculated.

21. *Ps. radicicola* from *Vicia faba*, Horse Bean. Colony on ash-maltose-agar. X 100.



22. *Ps. radicicola* from *Vicia villosa*, Hairy Vetch. Colony on ash-maltose-agar. X. 200.

Some inoculated seeds were immediately plated in ash-maltose-agar, and the remainder were dried in folds of sterile cotton and cheese cloth at room temperature and stored in sterile deep Petri dishes. For subsequent platings, seeds were placed in sterile Petri dishes in about a c.cm. of sterile distilled water, and allowed to remain thus fifteen to twenty minutes, the seeds being moved about occasionally with sterile forceps, the plates being then poured with ash-maltose-agar of the same stock in every case as was used for the first plating.

In this manner, desiccation on beans, peas, and red clover seed was observed, the results appearing in the table following:—

Number of Bacteria Alive on Seeds After Varying Periods of Drying.

PEAS.

		Before drying.	After 2 days,	After 6 days.	After 13 days.	After 228 days.
Seed + Culture	1	4,045	38	28	6	0
	2	6,487	59	21	28	0
	3	26,711	12,364	20	17	0
	4	9,799	636	152	14	0
Seed	1	382	.....	54	14	0
	2	191	.....	47	21	1
	3	719	.....	63	32	0
	4	954	.....	87	19	2
Sterile seed + Culture	1	41,594	604	65	0	0
	2	6,754	446	18	0	0
	3	2,823	259	34	0	0
	4	14,639	708	103	0	0
Sterile seed	1	0	0	0	0	0
	2	2	1	2	0	0
	3	0	0	0	0	0
	4	0	0	0	1	0

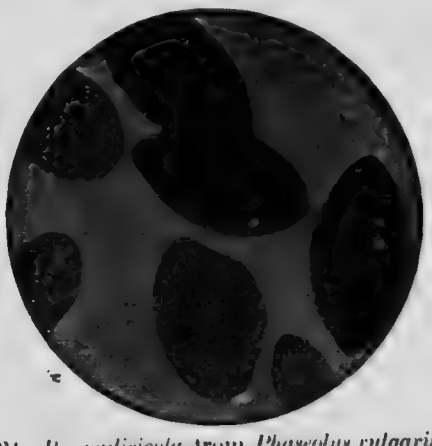
BEANS.					RED CLOVER.			
Before drying.	After 24 hrs.	After 6 days.	After 14 days.	After 223 days.	Before drying.	After 24 hrs.	After 6 days.	After 216dys.
7,085	5,787	2,989	1,372	0	477	53	50	0
37,333	.....	.....	5,215	mass on seed	21,636	40	43	0
14,437	13,582	3,243	2,874	0	.....	39	200	0
31,066	31,036	2,035	1,963	0	381	59	49	0
35	.....	21	.....	3 + mass on seed	69	.....	0	0
56	.....	15	.....	1	3	.....	0	0
41	.....	.....	.....	3	18	.....	0	0
28	.....	.....	.....	3	8	.....	0	0
29,065	0	.....	0	.....	.....	.....	.....	.....
27,602	0	.....	0	.....	.....	.....	.....	.....
34,782	0	.....	0	.....	.....	.....	.....	.....
26,056	0	.....	0	.....	.....	.....	.....	.....
0	0	.....	0	.....	.....	.....	.....	.....
0	0	.....	0	.....	.....	.....	.....	.....
0	0	.....	0	.....	.....	.....	.....	.....
0	0	.....	0	.....	.....	.....	.....	.....

A duplicate series of determinations were made with closely similar results.

Desiccation on Glass. From the same dilution of culture in each case as was used for inoculating seed sterile cover slips, one-half inch circles, received a two m.m. loop of the culture suspension. Some slips were plated in ash-maltose-agar immediately; the remainder were placed in sterile Petri dishes and were allowed to dry spontaneously at room temperature. For plating, the slips were removed to another sterile Petri dish with sterile forceps, flooded with sterile water, and after 15 to 20 minutes the plates were poured with ash-maltose-agar.

In every case the plate cultures of cover slips plated immediately on spreading showed more than 200 colonies—in some cases several thousand. In no case, however, did any colonies develop in plate cultures made 24 hours after the covers first received the culture.

23. *Ps. radicicola* from *Phaseolus vulgaris*, Garden Bean. Colony 12 days old on ash-maltose-agar. X 200.



24. *Ps. radicicola* from *Phaseolus vulgaris*, Garden Bean. Group of colonies on ash-maltose-agar. X 200.

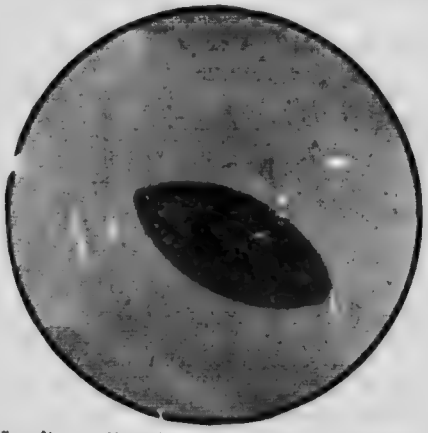
Desiccation on Paper. Pieces of filter paper of uniform size and shape were soaked in the suspension of culture prepared for inoculating seed; some pieces were plated immediately in ash-maltose-agar, the remainder being distributed in sterile Petri dishes and allowed to dry spontaneously at room temperature. For subsequent platings the pieces were flooded with sterile water a few minutes before pouring the plates, the pieces being moved about at intervals with sterile forceps. Colony counts showed that not more than 0.03% of the bacteria survived for 2 hours after being placed on the papers.

A sample of sweet pea seed supposed to be inoculated with commercial culture of the appropriate organism for this species of plan

was examined, and of eight seeds placed in ash-maltose-agar only one colony in one of the eight plates showed a characteristic staining reaction and cultural characters for *Ps. radicicola*.

The results obtained are in accord with the observations of other investigators that *Ps. radicicola* is especially susceptible to desiccation under these conditions.

A few observations were made upon the viability of *Ps. radicicola* in the dried nodule. It has always been our practice to preserve portions of plants and roots at the time of isolation of cultures, using the ordinary botanical plant presses. In plating from these dried specimens, the nodules were removed, washed in mercuric chloride solution, 1-1,000, and placed in sterile water until the nodule was plump. The ordinary procedure for plating, as heretofore described, was then followed. In every case, when growth occurred, the colonies were typical, as were also the staining reactions.



25. *Ps. radicicola* from *Lathyrus sylvestris*, Flat Pea. Colony 17 days on ash-maltose-agar. X 100.

26. *Ps. radicicola* from *Robinia pseudacacia*, Black Locust. Colony 22 days on ash-maltose-agar. X 100.

The results of these observations follow:—

Viability of Ps. radicicola in Dried Nodules.

Host Plant.	Bacteria alive after		Relative number of colonies.
	Years.	Days.	
Vicia Faba	0	137	Very many.
Vicia Faba	2	33	Few.
Medicago sativa	0	66	Very many.
Medicago sativa	0	93	Very many.
Glycine hispida	0	98	Many.
Caragana frutescens	2	131	Few.
Caragana frutescens	2	159	Few.
Trifolium pratense	2	138	Few.
Trifolium pratense	2	166	Few.

From these results, it would seem possible that a few individuals are able to withstand desiccation under this condition for long periods of time.

PREPARATION AND DISTRIBUTION OF CULTURES FOR INOCULATING SEED.

The preparation and distribution to farmers of cultures for inoculating seed was begun in the spring of 1905, and has been continued each season since. These cultures are sent in two-ounce square glass bottles, known in the trade as French squares, on ash-maltose-agar, prepared in the proportion of water 100, agar 1, maltose 0.5, ash 0.5. The bottles are filled about half full of the medium, plugged with cotton wool, sterilized, and allowed to solidify in an inclined position. Inoculation on the inclined agar surface is made from pure laboratory cultures. Growth is rapid at 25° C., and fairly abundant at 20° C. After a few days a copious mucilaginous or slimy growth covers the surface of the agar and gravitates to the bottom of the incline. In this condition the culture contains an ample number of bacteria to inoculate sixty pounds of seed. Plate culture determinations have shown that the ordinary cultures, when ready for distribution, contain from ten million to more than five billion living bacteria.

Before sending the culture the cotton stopper is replaced by a flamed cork, a sheet of directions is wrapped round the bottle and enclosed in a paper mailing case. See Fig. 45.

A copy of the directions sent with cultures follows:—

DIRECTIONS FOR INOCULATING SEED WITH NITROGEN-GATHERING BACTERIA.*

Each legume requires a different culture.

This bottle contains bacteria sufficient for inoculating sixty pounds

of.....seed.

All the culture may be used on lees seed without harm.

The culture is good for the season of 19.....

This culture is sent you with the understanding that you will use it as directed and report to us the result of your experiment. Follow directions carefully or failure may result.

1. Cover the seed with water and let it soak two hours.

2. Drain off the water. This may be done by heaping the seed on a cloth on the ground, or Nos. 1 and 2 may be done in a grain bag.

* Hereafter these directions will be modified to avoid the "sticky" seed caused by application of the sugar.

3. Mix one pound of dry granulated sugar with each bushel of the wet seed, and let the seed stand thus over night.

4. Next day pour a little clean cold water into the bottle of culture, shake until the jelly is well broken up, pour it over the seed and mix *thoroughly*.

5. Plant at once, just as you would uninoculated seed. If the seed is too wet and sticks together, spread it out in a shady place for about ten minutes. It should be neither wet nor dry, but as moist as it can be handled. In case of a mixture of clover with other seed, it is advisable to treat the clover separately, mixing just before sowing.

6. Do not open the bottle until you are ready to treat the seed, and do not treat more seed at one time than can be sown in a day.

7. At least a small plot should be planted with untreated seed for comparison, and this should be planted first.

8. Send us in the empty bottle with your name and address, a small sample of the inoculated seed for further laboratory tests.

9. After the seedlings are one month old, search for nodules, "little bunches," on the roots. Examine for nodules again after three months. During the season note number and size of nodules and vigor of plant growth from treated and untreated seed.

The price of each culture is twenty-five cents.

Originally the farmer was directed to shake the culture up thoroughly with a pint of water, mix the liquid with the seed and sow at once. It was found, however, during the last year that if the seed was soaked a few hours, and sugar added, the moisture on the seed was conserved, and the deleterious effect of desiccation of the bacteria before the seed was sown was largly overcome. That large numbers of the bacteria actually did enter the soil in the living condition is shown by the following:

Examination of Seed Samples Returned.

As noted in the directions, a request was made that recipients of the cultures the season just past send us, in the empty culture bottles, a small sample of the inoculated seed.

As soon as the sample was received, two average seeds were selected from the sample, placed in sterile Petri dishes, flooded with sterile water, allowed to stand 15 or 20 minutes, and the plates poured with ash-maltose-agar. The plates were incubated at room temperature, counts being made on the tenth day. At the time of making the count smears were made and stained with saturated alcoholic gentian violet. When only comparatively few colonies were present, these smears were made from single colonies. Where the colonies were very numerous, the smears were made by drawing the sterile loop through or over the surface of the medium.

In all, there were plated two seeds from each of 227 samples of different seeds, the number of samples of each kind being as follows: Alfalfa, 152; red clover, 52; alsike clover, 11; field peas, 7; sweet peas, 5.

In all but 25 of the 454 plates, stains with saturated alcoholic gentian violet showed typical reactions for *Ps. radicicola* and in 55 plates flagella were demonstrated.

The number of bacteria per seed, as indicated by the colony counts, is shown in the following table:—

Number bacteria per seed.	Samples.	Per cent.
None or stain negative	25	5.56
Less than fifty	91	20.04
Between 50 and 500	71	15.63
500 to 10,000	115	23.12
10,000 to 20,000	47	10.35
20,000 to 40,000	57	12.33
Over 40,000	36	8.14
Too numerous to count	12	2.64

From these results it would appear that a very ample number of bacteria go into the ground with the seed and in a vigorous condition.

27. *Ps. radicicola* in section of nodule from *Vicia villosa*, Hairy Vetch. Fixed in chrome-acetic acid and stained with Anilin-safranin-gentian violet. X 1,000.

28. *Ps. radicicola*. Section of nodule from *Vicia villosa*, Hairy Vetch, prepared as in 27. X 1,000.

This method of distributing cultures to farmers offers several advantages over the method of distribution by commercial houses. In most, at least, of the latter, the culture contains comparatively few of the organisms required, being accompanied with the appropriate chemical salts for making up a nutrient culture medium. In the hands of the farmer, the culture is certain to become contaminated, with the result that the *Ps. radicicola* may be inhibited or even entirely destroyed by the growth of foreign organisms. In the method followed in this laboratory, the bacteriologist assumes the responsibility for preparation

of the nutrient medium, and the propagation of the culture, and all the farmer has to do is to apply the bacteria to the seed.

We give herewith a brief resumé of results from the use of cultures for the past four seasons, as shown by farmers' reports. During 1905, 246 cultures were sent out, 134 reports being returned.

Crop.	Total No. of reports received.	Inoculation successful, with increased growth of crop.	Organisms already present in the soil.	No advantage from inoculation.
Lucerne or alfalfa	59	43	1	15
Red Clover	47	31	1	15
Peas	12	7	1	4
Beans	9	5		4
Alsike Clover	2	1		1
White Clover	1	1		
Vetch	3	2		1
Soy Bean	1	1		
	134	91	3	40

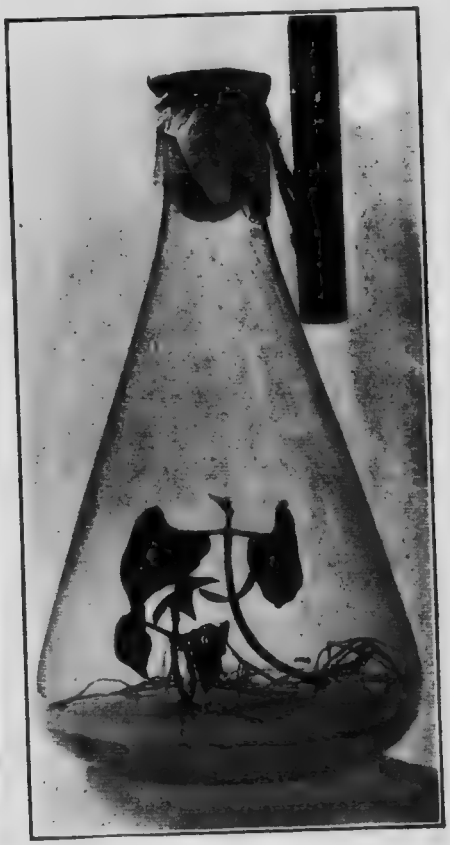
During 1906, 375 cultures were distributed; 120 reports were returned, 72 showing a benefit to the crop and 48 of no apparent benefit.

Province.	Crop Grown.		Result.	
			Benefit.	No Benefit.
Ontario	Alfalfa	12	9	3
	Red Clover	14	10	4
	Peas	14	9	5
	Beans	11	6	5
	Soy Beans	3	2	1
	Alsike	1	1	
	Vetch	1	1	
	Sweet Pea	1	1	
Alberta	Alfalfa	13	6	7
	Red Clover	3	1	2
	Peas	1	1	
Saskatchewan	Alfalfa	1		1
	Red Clover	2		2
	Peas	1	1	
	Beans	1		1
	Vetch	1		1
Manitoba	Alfalfa	2	2	
	Red Clover	8	6	2
Quebec	Alfalfa	3	3	
	Red Clover	1	1	
	Alsike	1	1	
United States	Alfalfa	5	3	2
	Red Clover	4	1	3
	Peas	3	2	1
	Beans	2	1	1
	Soy Beans	6	1	5
	White Clover	2	2	
	Vetch	1		1
New Brunswick	Red Clover	2	1	1

29. *Ps. radi icola.* Section of nodule from *Trifolium pratense*, Red Clover. Stained as in 27. X 1,000.

30. *Ps radicicola.* Section of nodule from *Trifolium pratense*, Red Clover. Stained as in 27. X 1,000. Compare with No. 12.

31. *Lathyrus odoratus*, Sweet Pea, showing the method of germinating sterile seeds under sterile conditions. These seedlings are ready to transplant into flasks.



32. *Phaseolus vulgaris*, Garden Bean, growing on ash-maltose-agar in 1½ L. Erlenmeyer flask.

During the season of 1907, there were distributed 372 cultures, and reports were available from 124. The summary of these is shown:—

Province.	Alfalfa.		Red Clover.		Alsike Clover.		Peas.		Beans.		Sweet Pea.	
	Benefit.	No Benefit.	Benefit.	No Benefit.	Benefit.	No Benefit.	Benefit.	No Benefit.	Benefit.	No Benefit.	Benefit.	No Benefit.
Ontario	36	28	6	11	2	....	2	2	1	....	....	....
New Brunswick	2	....	1	....	....	....	....	....	....	....	....	....
Saskatchewan	1	2	....	....	....	....	....	....	....	....	....	....
Alberta	1	1	....	....	....	....	....	....	....	....	....	....
P. E. Island	2	....	....	....	....	....	....	....	....	....	....	....
British Columbia	1	2	....	2	....	....	....	....	....	....	1	1
Quebec	3	1	....	....	....	....	....	....	....	1	1	....
Manitoba	1	1	....	1	....	....	....	1	....	....	....	....
Nova Scotia	1	....	2	....	....	1	....	....	2	....	....	....
Indiana, U.S.A	....	1	....	....	....	....	....	....	....	....	....	....
England	....	....	....	1	....	....	....	....	....	....	....	....
Ohio, U.S.A	....	....	....	....	1	....	....	....	....	....	....	....
Total	48	36	9	15	3	1	2	3	3	1	2	1

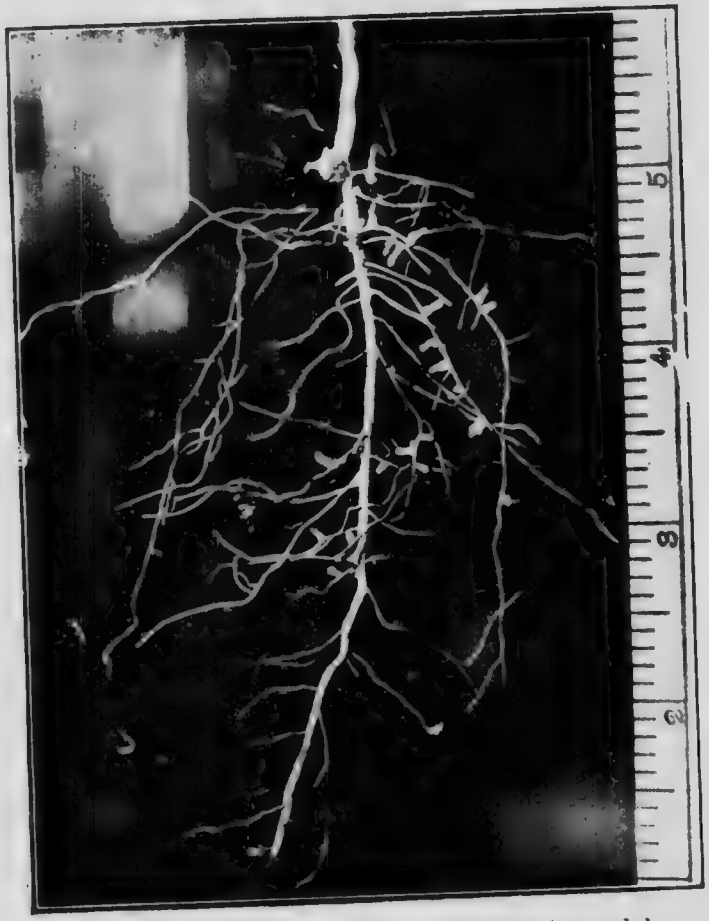
Total showing beneficial results, 54%.

Total showing no apparent benefit, 46%.

The distribution of cultures was continued during the season just passed, and a total of 2,113 cultures was sent, as noted in the table:—

Province.	Alfalfa.	Red Clover.	Beans.	Alsike Clover.	Peas.	Sweet Peas.	Vetches.
Ontario	1,236	319	31	68	108	17	4
Quebec	49	9	....	2	....	1	....
Nova Scotia	17	9	2	3	8	....	1
New Brunswick	12	3	....	....	1	....	....
P. E. Island	11	5	....	2	1	....	....
Newfoundland	1	....	....	....	....	....	....
Manitoba	16	3	....	1	....	....	....
Saskatchewan	11	1	....	1	2	....	....
Alberta	35	2	1	....	9	....	....
British Columbia	39	11	3	7	2	2	....
United States	3	12	....	2	7	3	....
England	2	....	....	....	1	....	....
Mexico	2	2	....	2	1	....	1
Sweden	....	1	....	....	1	....	1
Total	1,434	377	37	88	141	23	7

Also two cultures for yellow trefoil, and four for sweet white clover were sent.



33. Root of *Lathyrus odoratus*, Sweet Pea, showing nodules.

34. Portion of root of *Medicago sativa*, Alfalfa, showing nodules. Natural size.

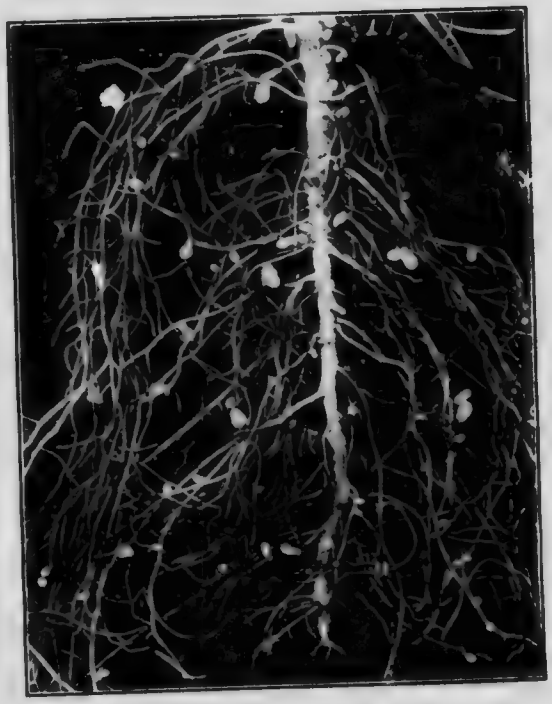
33. Root of *Lathyrus odoratus*, Sweet Pea, showing nodules.

34. Portion of root of *Medicago sativa*, Alfalfa, showing nodules. Natural size.

35. Root of *Pisum sativum*, Garden Pea, showing nodules.

36. Root of *Glycine hispida*, Soy Bean, showing nodules.

37. Root of *Trifolium pratense*, Red Clover, showing nodules. Natural size.



38. Root of *Vicia villosa*, Hairy Vetch, showing nodules Natural size.

39. Viability flask of Jena glass. Capacity about 50 c. cm.

40. Viability flask of Jena glass. Capacity about 100 c. cm. The long neck may be sealed after growth is abundant. When it is desired to transplant, the tube may be cut off, and resealed in the flame. There is ample length to repeat this several times if necessary.

The results of the season's inoculation, as seen from a summary of reports received, are shown in the table. The plus columns indicate the number of reports in which the farmer stated a positive beneficial result from the inoculation. The minus columns include all others. In many cases, the crop experimented with had been grown on the same ground previously, and the soil was probably already inoculated. Two farmers thought the inoculation was harmful to the crop.

Results of Seed Inoculation. 1908.

	Alfalfa.		Red Clover.		Alsike Clover.		Peas.		Beans.		Sweet Peas.	
	+	—	+	—	+	—	+	—	+	—	+	—
Ontario....	261	140	43	42	9	5	9	16	4	4		1
	65%	35%	50.5%	49.5%	.....	.....	.....	.....	.....	.....	.....	.....
B.Columbia	11	3	5	1	.....	.....	.....	.....	.....	.....	.....	.....
	78.5%	21.5%	83.3%	16.7%	.....	.....	.....	.....	.....	.....	.....	.....
Alberta....	10	8	1	.....	.....	.....	2	.....	.....	.....	.....	.....
	55.5%	44.5%	100%	.....	.....	.....	.....	.....	.....	.....	1	.....
Quebec....	10	6	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	62.5%	37.5%	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
P.E.Island	4	1	1	.....	.....	.....	.....	.....	.....	.....	.....	.....
	80%	20%	100%	.....	.....	.....	1	.....	.....	.....	.....	.....
Sask.....	3	1	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
	75%	25%	.....	.....	.....	.....	.....	.....	.....	.....	.....	.....
Nova Scotia	4	3	2	1	1	.....	1	1	.....	.....	1	.....
	57+%	43—%	67.7%	33.3%	.....	.....	.....	.....	.....	.....	.....	.....
New Bruns.	3	1	1	.....	.....	.....	.....	.....	.....	.....	.....	.....
	75%	25%	100%	.....	.....	.....	.....	.....	.....	.....	.....	.....
Manitoba..	3	2	2	.....	.....	.....	.....	.....	.....	.....	.....	.....
	60%	40%	100%	.....	.....	.....	.....	.....	.....	.....	.....	.....
U. S. A....	.....	.....	.....	1	.....	.....	1	.....	.....	.....	.....	.....
	.....	.....	.....	100%	.....	.....	.....	.....	.....	.....	.....	.....
Total....	309	165	55	45	10	5	14	17	4	4	5	1
	65%	35%	55%	45%	66.7%	33.3%	44%	55%	50%	50%	83.3%	16.7%

Following is the form of blank report sent to recipients of cultures:

DEAR SIR,—

Below is a blank for reporting the results of your experiment with nodule forming bacteria for legumes. We will esteem it a favour if you will fill out this report and return it to us promptly in the enclosed envelope.

Crop seeded .....
Character of land .....
Amount of treated seed .....
Amount of untreated seed .....

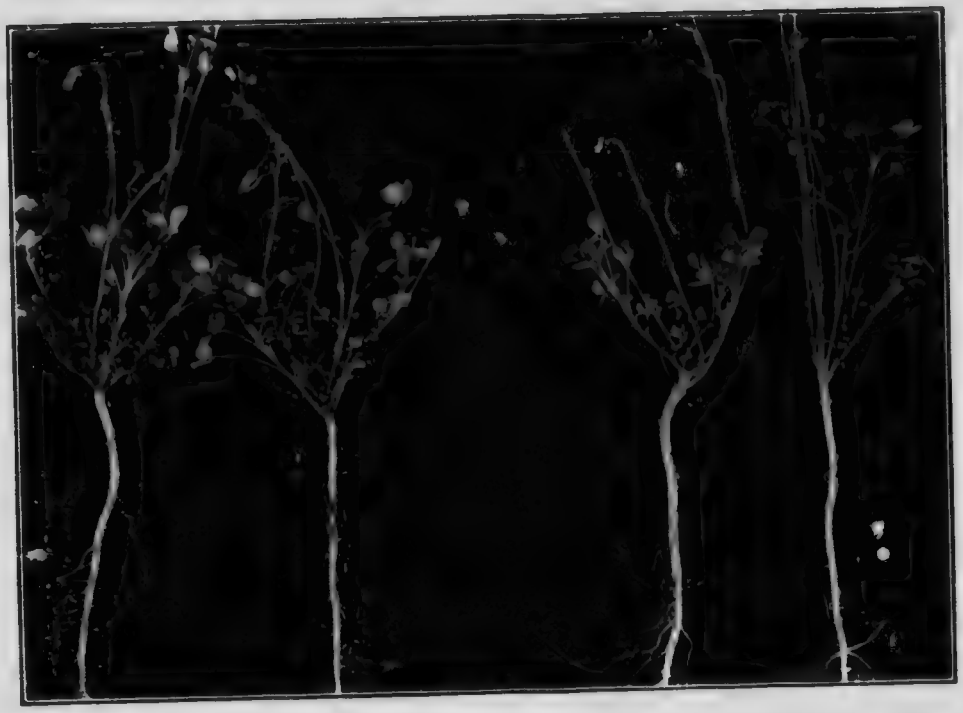
	With Culture.	Without Culture.
Area of land planted		
Nodules present or absent at one month		
Few or numerous nodules after three months		
Vigor of plants after three months		

Do you think your crop has been benefited by the culture? .....
Do you expect to use similar cultures next season? .....
Weather conditions during the season? .....
Remarks:—

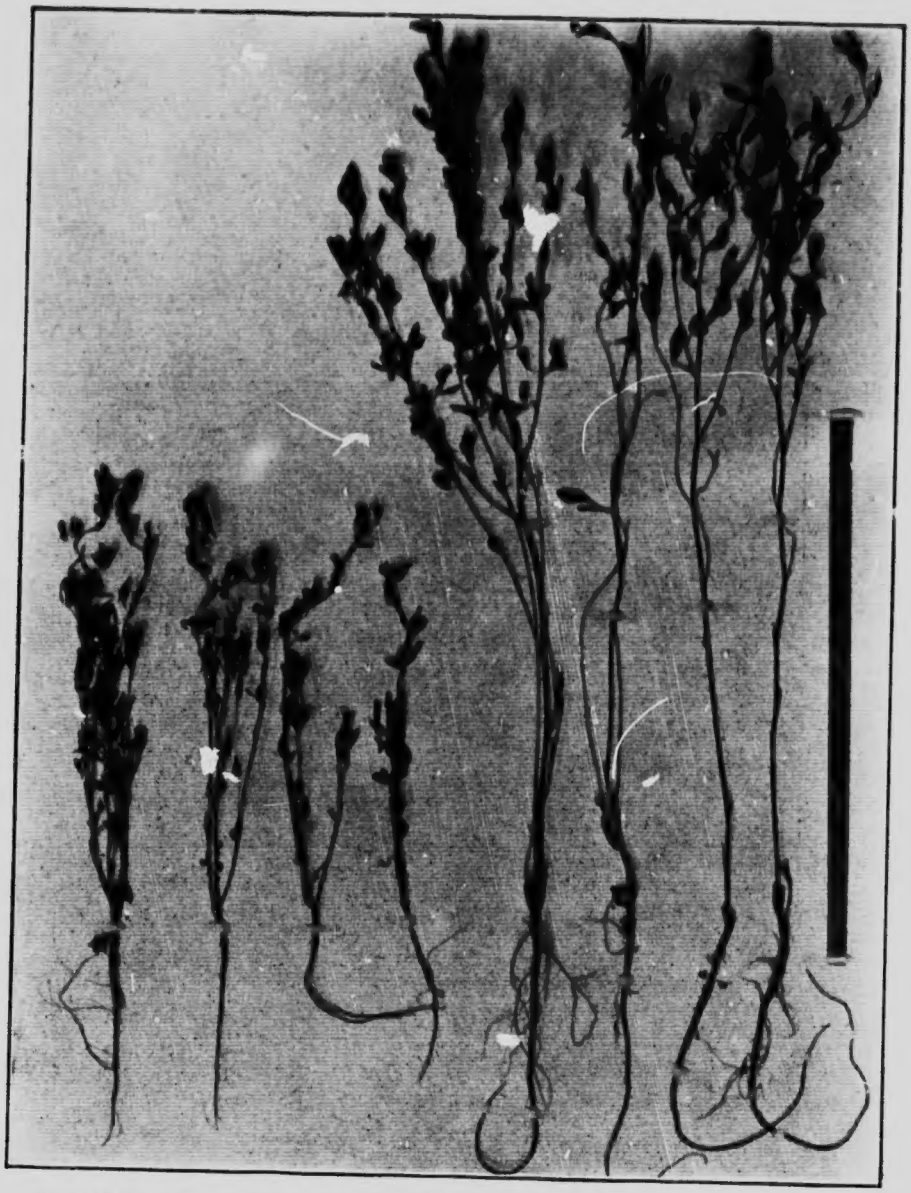
Your name .....
Post Office .....
Province .....

41. Alfalfa plants one month old, from inoculated seed. Berlin, Ont.

42. Alfalfa plants one month old from uninoculated seed sown in the same field and at the same time as No. 41. Berlin, Ont.



43. Average plants from inoculated and uninoculated portions respectively of a field of alfalfa. Ontario Agricultural College.



44. Alfalfa plants from inoculated and uninoculated seed. Pictou Landing, Nova Scotia.

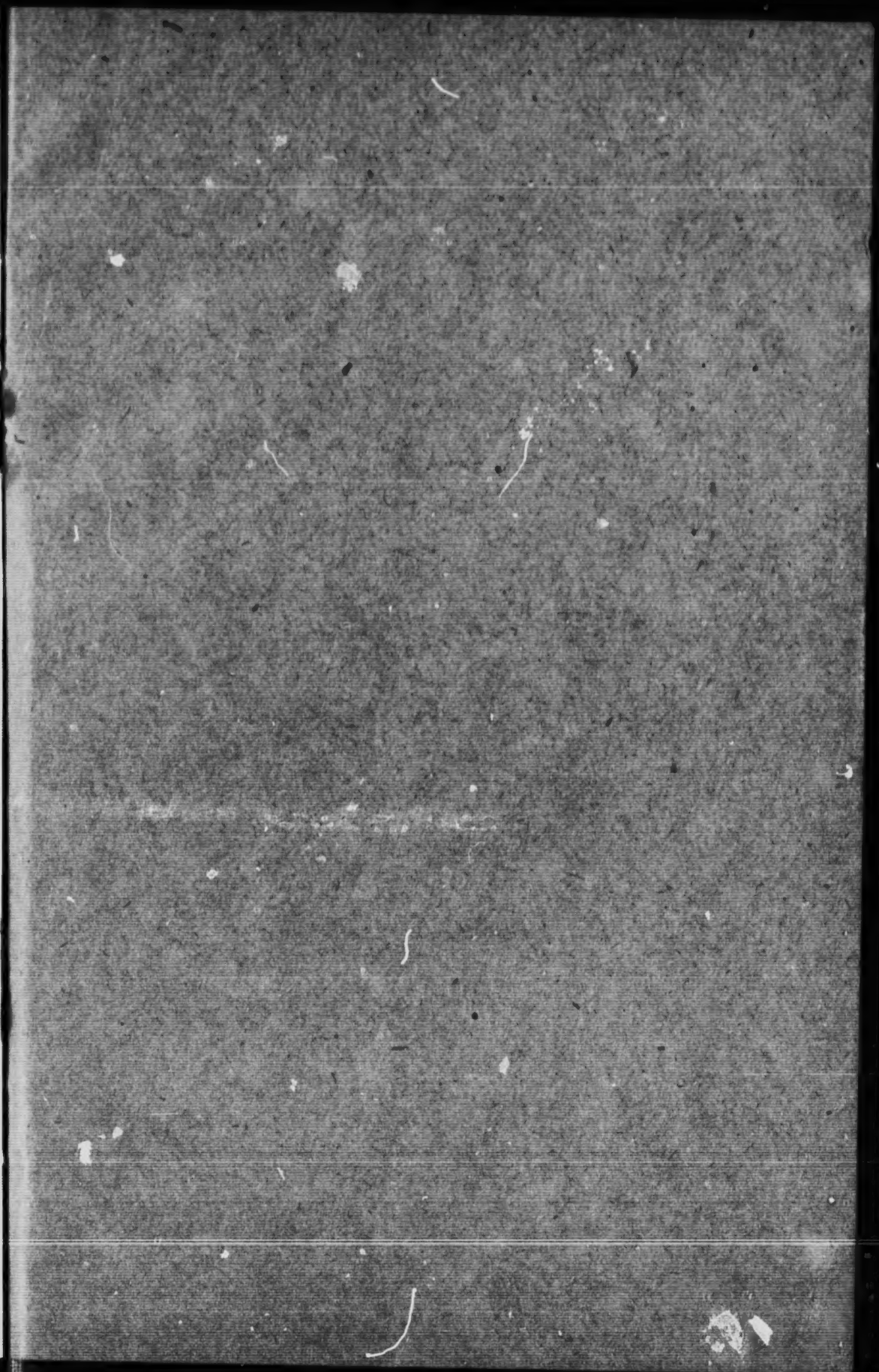
To briefly summarize:—A total of 2,113 cultures were distributed, and reports were returned from 634, or 31.7%. Of these, positive beneficial results were stated in 397, or 62.6%, and no benefit was apparent in 237, or 37.4%

In reply to the query, 337, or 53%, experimenters expressed a wish to secure similar cultures next season, this number including many who reported no apparent benefit this season.

45. The cultures for inoculating seed are sent out on ash-maltose-agar in 2 oz. French squares, enclosed with directions for use, in a paper mailing case.

It is the intention of the Department of Bacteriology to distribute these cultures during 1909. As heretofore, a price of 25 cents is affixed for each bottle of culture sufficient for 60 pounds or less of seed, and each farmer is expected to submit a report of his work at the end of the season.

LABORATORY OF BACTERIOLOGY,
GUELPH, December, 1908.



LIST OF BULLETINS

PUBLISHED BY THE ONTARIO DEPARTMENT OF AGRICULTURE, TORONTO.

Serial No.	Date.		Title.	Author.
133	Dec.	1903	Present Condition of San Jose Scale in Ontario	Wm. Lochhead.
134	June	1904	Hints in Making Nature Collections in Public and High Schools	W. H. Muldrew.
135	June	1904	The Cream-Gathering Creamery	H. H. Dean. J. A. McFeeters.
136	Aug.	1904	Some Bacterial Diseases of Plants Prevalent in Ontario	F. C. Harrison. B. Barlow.
137	Aug.	1904	A Bacterial Disease of Cauliflower and Allied Plants	F. C. Harrison.
138	Feb.	1905	The Composition of Ontario Feeding Stuffs	W. P. Gamble.
139	Feb.	1905	An Experimental Shipment of Fruit to Winnipeg	J. B. Reynolds.
140	Feb.	1905	The Results of Field Experiments with Farm Crops	C. A. Zavitz.
141	April	1905	Gas-Producing Bacteria and Their Effect on Milk and its Products	F. C. Harrison.
142	May	1905	Outlines of Nature-Study	Wm. Lochhead.
143	June	1905	Dairy School Bulletin	Dairy School.
144	June	1905	Apple Culture	H. L. Hutt.
145	June	1905	Butter Preservatives	H. H. Dean. R. Harcourt.
146	Nov.	1905	Uses of Fruits, Vegetables and Honey	
147	Feb.	1906	Fruits Recommended for Ontario Planters	Fruit Ex. Stations.
148	Mar.	1906	Experiments with Nodule-forming Bacteria	F. C. Harrison. B. Barlow.
149	July	1906	The Swine Industry in Ontario	
150	Aug.	1906	The Common Fungus and Insect Pests of Growing Vegetable Crops	Wm. Lochhead T. D. Jarvis
151	Oct.	1906	Farm Poultry (Revised Nov., 1907)	W. R. Graham
152	Dec.	1906	Gardening for Schools	S. B. McCready
153	Feb.	1907	Fertilizers and their Use	R. Harcourt.
154	Feb.	1907	Insecticides and Fungicides	R. Harcourt. H. L. Fulmer.
155	Feb.	1907	Farm Forestry (Second Edition, Dec., 1907)	E. J. Zavitz.
156	Mar.	1907	Tillage and Rotation	W. H. Day.
157	Mar.	1907	Remedies for the San Jose Scale, San Jose Scale Act	
158	June	1907	Insects and Fungus Diseases Affecting Fruit Trees, Revised Dec., 1907	C. J. S. Bethune. T. D. Jarvis.
159	July	1907	Milking Machines	H. H. Dean. S. F. Edwards.
160	July	1907	The Production, Care, and Uses of Milk	
161	Oct.	1907	The Sheep Industry in Ontario	
162	Dec.	1907	Breakfast Foods: Their Chemical Composition, Digestibility and Cost	R. Harcourt. H. L. Fulmer.
163	Mar.	1908	Incubation of Chickens	O.A.C. Staff.
164	Mar.	1908	Legume Bacteria	S. F. Edwards. B. Barlow.
165	Mar.	1908	Alfalfa or Lucerne	C. A. Zavitz.
166	June	1908	Bee-keeping in Ontario	
167	Oct.	1908	Mitchell-Walker Moisture Test	J. W. Mitchell. W. O. Walker.
168	Oct.	1908	The Perennial Sow Thistle and some other Weed Pests	J. E. Howitt.
169	Feb.	1909	Legume Bacteria: Further Studies of Nitrogen Accumulation in the Leguminosæ	S. F. Edwards. B. Barlow.